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**Analysis of the transcriptional regulation and molecular function of the Aryl
Hydrocarbon Receptor Repressor in human cell lines**

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AhR, aryl hydrocarbon receptor; AhRR, aryl hydrocarbon receptor repressor; ARNT, aryl hydrocarbon receptor nuclear translocator; B(a)P, benzo(a)pyrene; bHLH, basic helix-loop-helix; ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; HDAC, histone deacetylase; 3-MC, 3-methylcholanthrene; MitA, mithramycin A; MNF, 3'-methoxy-4'-nitroflavone; NaB, sodium butyrate; PAS, Per-Arnt-Sim; Sp, specificity protein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TSA, trichostatin A; XRE, xenobiotic-responsive element

Abstract

The aryl hydrocarbon receptor repressor (AhRR) is a member of the AhR signaling cascade, which mediates dioxin toxicity and is involved in regulation of cell growth and differentiation. The AhRR was described as a feedback modulator which counteracts AhR-dependent gene expression. We investigated the molecular mechanisms of transcriptional regulation of the human *AhRR* by cloning its regulatory DNA region located in intron I of the *AhRR*. By means of reporter gene analyses and generation of deletion variants, we identified a functional, 3-methylcholanthrene-sensitive XRE site. Chromatin immunoprecipitation analyses revealed that the AhRR binds to this XRE, displaying an autoregulatory loop of *AhRR* expression. In addition we show that an adjacent GC-box is of functional relevance for *AhRR* transcription, since blocking of this GC-box resulted in a decrease of constitutive and inducible *AhRR* gene activity. The differences in constitutive AhRR mRNA level observed in HepG2, primary fibroblast and HeLa cells are directly correlated with CYP1A1 inducibility. We show that the non-responsiveness of high AhRR-expressing cells towards AhR-agonists is associated with a constitutive binding of the AhRR to XRE sites of *CYP1A1*. Treatment with the histone deacetylase (HDAC) inhibitor sodium butyrate restored the responsiveness of *CYP1A1* in these cell lines, due to the dissociation of AhRR from the XREs. Furthermore, transient AhRR mRNA-silencing in untreated HeLa cells was accompanied by an increase of basal CYP1A1 expression, pointing to a constitutive role of the AhRR in regulation of *CYP1A1*. The functional relevance of the AhRR in high AhRR-expressing primary fibroblasts is discussed.

Introduction

The aryl hydrocarbon receptor (AhR) is a member of the basic Helix-Loop-Helix Per-ARNT-Sim (bHLH-PAS) protein superfamily which modulates gene transcription in response to developmental and environmental signals (Crews and Fan, 1999; Gu et al., 2000). It is the only known ligand-activated transcription factor in the family of PAS proteins and mediates the toxicity of xenobiotic compounds like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo(*a*)pyrene (B(*a*)P) and 3-methylcholanthrene (3-MC) (Denison and Nagy, 2003; Bock and Köhle, 2006). The molecular mechanism of AhR activation is well understood and described in several review articles (Mimura and Fujii-Kuriyama, 2003; Marlowe and Puga, 2005; Bock and Köhle, 2006). Briefly, in the inactive non-liganded form the AhR is located in the cytoplasm as a multiprotein complex consisting of two heat shock proteins 90, the immunophilin-like AIP/XAP2 and the cochaperone p23. Upon ligand binding this complex dissociates and the AhR translocates into the nucleus where it dimerizes with the AhR nuclear translocator (ARNT). The resulting heterodimer binds to xenobiotic responsive elements (XRE) which are DNA sequences located in enhancer regions of target genes and regulate their transcription. The best characterized AhR target gene is *CYP1A1* (Whitlock, Jr., 1999; Ma, 2001). Other typical targets for the activated AhR are genes encoding the phase I enzymes CYP1B1 and CYP1A2 and the phase II enzymes UDP-glucuronosyltransferases 1A1 and 1A6 (Bock and Köhle, 2006). Besides its regulatory role in drug-metabolism, there is a growing list of evidence that the AhR is involved in regulation of cell proliferation and differentiation (Nebert et al., 2000; Marlowe and Puga, 2005; Bock and Köhle, 2006).

The AhR repressor (AhRR), another member of the bHLH-PAS protein family, was discovered in 1999 and identified as a new AhR-regulated gene (Mimura et al., 1999). The function of the AhRR was described as a negative feedback modulator of the AhR pathway, doing this by competing with the AhR for ARNT- and XRE-binding, thereby blocking AhR-dependent gene expression (Mimura et al., 1999). The *AhRR* gene was detected in several

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species including fish, rat, mice and humans (Tsuchiya et al., 2003a; Korkalainen et al., 2004; Yamamoto et al., 2004; Evans et al., 2005; Bernshausen et al., 2006; Nishihashi et al., 2006; Roy et al., 2006). Expression analysis of AhRR mRNA in different organs of mice, rats and humans revealed a tissue-specific expression of the AhRR (Tsuchiya et al., 2003a; Korkalainen et al., 2004; Yamamoto et al., 2004; Bernshausen et al., 2006; Nishihashi et al., 2006). The same was observed in several human cell lines where AhRR transcripts display a cell type-specific expression pattern (Tsuchiya et al., 2003a). The molecular mechanisms behind the organ- and cell-specific differences in AhRR expression are still unknown.

So far, only few papers concerning transcriptional regulation of the *AhRR* have been published (Baba et al., 2001; Nishihashi et al., 2006). Several XRE sites and three GC-boxes of functional relevance have been identified in the 5'-upstream region of the mouse *AhRR* gene. Up to now, no experimental data were reported for the transcriptional regulation of the human *AhRR*.

In this study we investigated the transcriptional regulation and the molecular function of the AhRR in human cell lines. We cloned regulatory regions within intron 1 of the *AhRR* gene and analyzed the expression and function of the AhRR in different human cell lines: the AhR agonist responsive cell line HepG2 and the non-responsive primary fibroblast and HeLa cells. Our data identify functionally active regulatory sequences in intron 1 of the human *AhRR* and show that the AhRR is involved in the transcriptional regulation of the AhR-sensitive *CYP1A1* gene.

Material and Methods

Materials. Cell culture media and the chemicals B(a)P, 3-MC, Mithramycin A (MitA, trade name Plicamycin) and sodium butyrate (NaB) were purchased from Sigma-Aldrich (Munich, Germany). Restriction endonucleases, M-MLV reverse transcriptase and Fugene 6 transfection reagent were supplied by Roche (Mannheim, Germany). *Taq* polymerase, RNeasy Mini Kit, Plasmid Midi Kit and SYBR Green PCR Kit were obtained from Qiagen (Hilden, Germany). Dual luciferase reporter assay kit and the *pGL3-basic* vector system were purchased from Promega (Mannheim, Germany). The DNA Blunting Kit was delivered by TaKaRa (Japan), the competent TOP10 *E.coli* cells by Invitrogen (Karlsruhe, Germany). Protein A Sepharose, anti-AhR and anti-ARNT were obtained from Biomol (Hamburg, Germany), the Protease Inhibitor Cocktail III from Calbiochem (Darmstadt, Germany). Oligonucleotides and siRNAs were obtained from MWG-Biotech AG (Ebersberg, Germany). AhRR antiserum was prepared by immunising rabbits against the specific peptide sequence CQFQGKLFKFLFGQKKK.

Cell Culture and Treatment. The human hepatocarcinoma cell line HepG2 and the human cervical adenocarcinoma cell line HeLa were cultured in RPMI 1640 medium with 3,7% (w/v) NaHCO₃ and 10% (v/v) fetal calf serum. Dermal primary fibroblasts, isolated from human foreskin, were cultured in MEME adjusted with 2mM glutamine and 10% (v/v) fetal calf serum. For RT-PCR analyses, reporter gene assays and siRNA experiments 2x 10⁵ cells/well were seeded and maintained under standard conditions at 37°C and 6.5% CO₂. In general, treatment of the cells was performed 24h after seeding. 3-MC and MNF were dissolved in DMSO, B(a)P in tetrahydrofurane. NaB and MitA were dissolved in sterile water. Exposure of cells to either 3-MC, MNF or B(a)P occurred in a final concentration of

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10 μ M. NaB or MitA were used in a concentration of 10mM or 150nM, respectively. Time-points of treatments are indicated in the figure legends.

Quantitative Real-Time PCR. Total RNA was isolated from cells using the RNeasy Mini Kit, according to the manufacturer's instructions, including *DNase I* digestion. Subsequently, 1 μ g of total RNA was reverse transcribed in a final volume of 40 μ l using M-MLV reverse transcriptase. Quantitative real-time PCR was carried out in a LightCycler instrument (Roche) using the QuantiTect SYBR Green PCR Kit and 2 μ l of each cDNA sample. The relative copy-numbers of AhRR and CYP1A1 transcripts were calculated by the LightCycler software and normalised against the relative amount of β -actin transcripts. Each sample was analysed in triplicate. The oligonucleotide sequences and annealing temperatures for amplification of human CYP1A1, AhRR, and β -actin were described previously (Fritsche et al., 2005).

Chromatin Immunoprecipitation (ChIP) Assays. Confluent flasks of HepG2, HeLa or primary fibroblast cells ($\sim 2\text{-}3 \times 10^7$) were rinsed with phosphate-buffered saline (PBS) and crosslinked with 1% formaldehyde for 10min at room temperature. Crosslinking was stopped by adding glycine to a final concentration of 0.125mM. Cells were rinsed twice with ice-cold PBS, scraped in 5ml ice-cold PBS and pelleted by centrifugation. Pellets were washed with PBS and resuspended in 1ml lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1 and complete protease inhibitor cocktail) and incubated for 30min on ice. Lysates were sonicated (3-4 x 10 sec) with a Branson Sonifier 250 to produce DNA-fragments between 0.2kb and 1.5kb in size. Lysates were centrifuged for 20min at 4°C, supernatants were aliquoted. Nine volumes of dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 150mM NaCl, and complete protease inhibitor cocktail) were added to supernatants. Samples were pre-cleared by adding 35 μ l of a 50% protein A sepharose slurry, blocked with sheared salmon sperm DNA and BSA. After 2h of incubation

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at 4°C on a rotating wheel, beads were pelleted and supernatants were transferred into new tubes. An appropriate amount of antibody was added and samples were incubated overnight at 4°C on a rotating wheel. Immunocomplexes were precipitated by adding 30µl of blocked beads. Beads were pelleted and washed sequentially with low salt buffer (0.1% SDS, 1.0% TritonX-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), high salt buffer (0.1% SDS, 1.0% TritonX-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 0.5M NaCl) and LiCl buffer (250mM LiCl, 1% NP-40, 1% Na-deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1). Finally, beads were washed twice in TE buffer. Immunocomplexes were eluted by adding 200µl elution buffer (1% SDS, 100mM NaHCO₃). Eluates were digested with proteinase K for 2h at 55°C, followed by a de-crosslinking step at 65°C overnight. DNA was purified by phenol-chloroform extraction, ethanol precipitated and resuspended in 25µl water. Aliquots of every ChIP sample were diluted 1-5 and 1-10. Subsequently, 5µl of each dilution were amplified in 35-40 PCR cycles, using *Taq* polymerase. Oligonucleotides used for detection of the XRE-bearing fragment of the human CYP1A1 gene were published previously (Hestermann and Brown, 2003). The oligonucleotides for amplification of the XRE-containing AhRR sequence (+17.388 to +17.711) were 5'-AACCGGATGGCGTCTCCGGTTC-3' and 5'-GAGCGCATCCCGTCTCCACC-3', the annealing temperature was 64°C.

Generation of AhRR Reporter Gene Plasmids. The reporter construct *pAhRR* was created by insertion of a 1025bp fragment of *AhRR* intron I (+16.873 to +17.897) into the *KpnI* / *XhoI*-site of the *pGL3-basic* vector. The *AhRR* fragment was amplified by PCR using the following linker-primer 5'-GACGGTACCCAGGCAGGACCCTGCTCTCCGG-3' and 5'-CTCTCGAGGGGACGCTGAGGACCACGAACCGT-3'. Deletion constructs of *pAhRR* were produced by endonuclease restriction and subsequent religation of the plasmids. For construction of *pAhRR-ΔI*, the *pAhRR* plasmid was digested with *BglII* and *PvuII*. The

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pAhRR-Δ2 construct was generated by cutting *pAhRR* with *KpnI* and *PvuII*. Digestion of *pAhRR* with *BglII* and *BssHII* lead to the *pAhRR-Δ3* deletion, and with *KpnI* and *BssHII* to the *pAhRR-Δ4* construct, respectively. The *pAhRR-Δ5* was produced by cutting *pAhRR-Δ2* with *BglII* and *BssHII*. After enzyme digestion, the plasmids were religated using the TaKaRa DNA Blunting Kit. The *pAhRR-mut* construct was synthesized by Slonomics BioTechnology GmbH (Puchheim, Germany). The core sequence GCGTG of XRE-3 was modified to TTCTC. All of the above mentioned reporter constructs were transformed into TOP10 *E.coli* cells using the heat-shock method. Subsequently, multiplied plasmids were recovered using the Qiagen plasmid midi kit.

Reporter Gene Assays. HepG2 cells (2×10^5 /well) were seeded into six-well plates and maintained overnight under standard conditions. Cells were transiently transfected with $2 \mu\text{g}$ /well of AhRR reporter constructs or empty *pGL3-basic* vector and 50ng /well of *pRL-TK* Renilla luciferase control plasmid by using the Fugene 6 transfection system. After 4h of incubation the transfection reagent/DNA mix was replaced by fresh full medium and cells were incubated overnight. Cells were treated with the indicated substances for 24h or 48h. The cell lysates were collected and luciferase activities of the *pAhRR* reporter plasmids were determined using the dual-luciferase assay system in a Multi-Bioluminat LB 9505C (Berthold, Germany). Firefly luciferase activity was normalized to renilla luciferase activity.

Transient RNA-Interference Experiments. For transient transfection of HeLa cells with AhRR siRNA, 2×10^5 cells/well were seeded into six-well plates and cultured overnight. The cells were transfected with 100nM of AhRR siRNA or non-silencing control sequences using the Fugene 6 reagent. The AhRR siRNA sequences were: sense 5'-GCAACGAUCGUGGACUAUC-3', antisense 5'-GAUAGUCCACGAUCGUUGC-3'. Efficiency of AhRR knock-down was tested by quantitative real-time PCR.

Bioinformatics. DNA sequence analyses were performed using the NCBI BLAST tools (<http://www.ncbi.nlm.nih.gov/BLAST/>) and a GCG-based software package, provided by the HUSAR Bioinformatics Lab (<http://genome.dkfz-heidelberg.de/>). In addition, putative transcription factor binding sites were analyzed using TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) and Tfsitescan databases (<http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl>). The nucleotide sequences used in this work are available via GenBank accession numbers NT_006576.15, AH010004 and NM_020731. In this study the beginning of human AhRR exon 1 is designated as base +1.

Statistics. All data are presented as mean \pm standard deviation. Data were analysed using ANOVA and Student's T-test (Excel). *P*-values below 0.05 were considered as significant.

Results

Identification of functional XRE sites in the human *AhRR* gene

To assess the regulatory activities of the human *AhRR* gene, we performed computer-based promoter analyses and identified four putative XRE sites located at position +16.959 (XRE-1), +17.143 (XRE-2), +17.507 (XRE-3) and +17.678 (XRE-4) (Fig. 1A). To examine the functional relevance of these sites, we then amplified a XRE-1 to -4 containing 1.025kb DNA fragment of intron 1 and fused it into the luciferase expression vector *pGL3-basic* (*pAhRR*). Treatment of *pAhRR* transfected HepG2 cells with 10 μ M of the AhR agonist 3-MC for 48h resulted in a 600- to 700-fold increase of luciferase activity in comparison to the basal activity of the empty vector *pGL3-basic* indicating that at least one of the four XREs is probably functional (Fig. 1B). In comparison to the respective solvent control, the *pAhRR*-driven luciferase activity was 7- to 8-fold enhanced after 3-MC treatment. In order to identify functional XRE motifs within this regulatory fragment we performed deletion analyses with *pAhRR*. Removal of the putative XRE-3 and -4 sites (Fig. 1B, *pAhRR- Δ 1*) resulted in a loss of basal and 3-MC induced luciferase activity. In contrast, the deletion of XRE-1 and -2 motifs (Fig. 1B, *pAhRR- Δ 2*) retained the responsiveness of the luciferase reporter gene towards solvent and 3-MC. The measured activities were within the same range as detected for *pAhRR*. These results indicate that either XRE-3 or XRE-4 mediate the activity of the luciferase reporter plasmids. Therefore, we generated two additional deletion constructs containing the putative XRE-3 or XRE-4 sites. Transfection of HepG2 cells with the *pAhRR- Δ 4* lacking XRE-3 resulted in a significant decrease of luciferase activities when compared to *pAhRR* or *pAhRR- Δ 2*, respectively (Fig. 1B). In contrast, deletion of XRE-4 had no influence on constitutive and 3-MC induced luciferase activity (Fig. 1B, *pAhRR- Δ 5*) indicating that only XRE-3 is of functional importance for AhRR promoter activity. To investigate the functional activity of XRE-3, the GCGTG core sequence was changed to TTCTC. As seen in figure 1B, this mutation (*pAhRR-mut*) resulted in a significant loss of basal and inducible luciferase

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activity. This result confirms the functional importance of XRE-3 in AhRR gene regulation. In order to confirm that the 3-MC induced luciferase expression is mediated by the activated AhR, AhR activity was pharmaceutically blocked by co-treatment of the transfected cells with the specific AhR antagonist 3'-methoxy 4'-nitroflavone (MNF) (Lu et al., 1995). In presence of MNF the 3-MC induced reporter gene activity was reduced to the level of solvent control (Fig. 1C). The data from the reporter gene analyses confirm that the XRE-3 site is functional as an inducible enhancer element.

Binding of AhR, ARNT and AhRR to XRE-3 of the human *AhRR* gene

In order to investigate whether the AhR, ARNT and the AhRR are functionally integrated in the constitutive and inducible expression of the human *AhRR* gene, we performed ChIP assays. As shown in figure 2, binding of AhR, ARNT and AhRR to the XRE-3 of the *AhRR* gene was detected in untreated HepG2 cells. Treatment of the cells with 10 μ M 3-MC for a period of 60 minutes led to an oscillating binding pattern of the three proteins. After 20 minutes of 3-MC exposure a strong XRE-binding of the AhRR protein was detected. At this time-point the AhR seemed to be displaced from the respective XRE. At later time-points we observed a strong AhR/XRE association, whereas the AhRR binding signal seemed to diminish. The results of the ChIP analyses suggest that the AhRR is involved in the regulation of its own transcription.

Involvement of a GC-box sequence in regulation of AhRR gene expression

As seen in figure 1A, sequence analysis of the cloned *AhRR* fragment revealed a GC-box sequence 8bp 5'-upstream of XRE-3. Therefore, we questioned if this sequence is of functional relevance for the transcriptional regulation of the *AhRR* gene. Luciferase assays disclosed that in the absence of any inducer, the activities of the *pAhRR*, *pAhRR- Δ 2* and *pAhRR- Δ 5* plasmids, which are GC-box proficient, were about 100-fold higher than the basic

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activity of the *pGL3-basic* vector (Fig. 1B). In contrast, the deletion constructs which lack the GC-box (Fig. 1B, *pAhRR-Δ1*, *pAhRR-Δ3*, *pAhRR-Δ4*) obtain significantly less constitutive luciferase activities. To confirm that these constitutive activities can be indeed attributed to the putative GC-box, we treated the *pAhRR-Δ5* harboring HepG2 cells with MitA. This chemical is known to bind to GC-rich DNA sequences, thereby inhibiting the binding of zinc-finger containing transcription factors like Sp1 (Blume et al., 1991; Nehls et al., 1993). As shown in figure 3, exposure of the transfected cells to MitA resulted in a significant decrease in constitutive luciferase activity of *pAhRR-Δ5*. In order to analyze if MitA treatment also influences the inducible luciferase activity, transfected cells were co-treated for 24h with 3-MC and MitA. In the presence of MitA, we observed a significant reduction of luciferase response of the reporter gene towards 3-MC (Fig. 3) suggesting a cooperative action of XRE-3 and GC-box sequences on the constitutive and inducible regulation of the human AhRR gene. This cooperative mode of action was verified by the finding, that the mutation of XRE-3 led to a significant reduction of constitutive and inducible luciferase activity (Fig. 1B, *pAhRR-mut*). To further support these findings, RT-PCR analyses were performed in HeLa cells which are known to express high constitutive levels of AhRR mRNA (Tsuchiya et al., 2003a). Treatment of these cells with MitA over a period of 12h led to a time-dependent decrease of AhRR mRNA (Fig. 4A). Three hours after MitA exposure, the measured AhRR expression was reduced to 50% of the control value, indicating a rapid turn-over of the AhRR mRNA in HeLa cells. After 12h of MitA treatment AhRR mRNA was reduced to 25% of the control value (Fig. 4A).

To address whether the decrease of AhRR mRNA is associated with changes of *CYP1A1* expression, we performed RT-PCR analyses. As seen in figure 4A the decrease of AhRR mRNA is accompanied by a gradual increase of *CYP1A1* transcription. 12h after MitA treatment, *CYP1A1* expression was 4- to 5-fold enhanced. Since HeLa cells are known to be non-responsive towards AhR agonists (Iwanari et al., 2002), we investigated whether MitA

treatment restores the responsiveness of *CYP1A1* towards 3-MC. The results show that co-treatment of HeLa cells with 3-MC and MitA led to a ~7-fold increase of *CYP1A1* gene expression, whereas the AhRR mRNA content was reduced to 20% of the control value (Fig. 4B). Exposure of the cells to 3-MC alone had no influence on AhRR or *CYP1A1* expression. Taken together, the PCR and reporter gene data support the supposition of a cooperative interaction between XRE- and GC-box motifs in regulation of AhRR transcription. Furthermore, the MitA experiments performed in HeLa cells strongly indicate an important role of AhRR in the control of *CYP1A1* expression.

The AhRR mediates the non-responsiveness of fibroblast and HeLa cells towards AhR agonists

HeLa cells and fibroblasts are considered as high AhRR-expressing cell lines and known to be non-responsive towards AhR agonists (Gradin et al., 1999; Iwanari et al., 2002; Tsuchiya et al., 2003a). In agreement with this data (Tsuchiya et al., 2003a), AhRR mRNA expression was 5-fold higher in fibroblast and 10-fold higher in HeLa cells compared to HepG2 cells (data not shown). In both cell lines the expression of *CYP1A1* remained unchanged after treatment with 3-MC or B(a)P. Our results disclose a possible role of the AhRR in modulating the constitutive and inducible *CYP1A1* expression. Therefore, we asked whether a transient down-regulation of AhRR mRNA might be associated with a modulation of *CYP1A1* expression. As shown in figure 5, transient silencing of AhRR mRNA was accompanied with a ~5-fold increase of constitutive and a ~20-fold increase of 3-MC-induced *CYP1A1* mRNA expression in HeLa cells. This result points toward a suppressive activity of the AhRR on *CYP1A1* expression in HeLa cells, possibly due to binding of the AhRR to XRE sites.

In order to test this hypothesis, we performed ChIP analyses using an anti-AhRR antibody. Untreated primary fibroblasts reveal a strong binding of the AhRR to the *CYP1A1* XRE (Fig. 6). Treatment of the fibroblasts with B(a)P did not affect the AhRR/XRE association. Since it

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was reported that the HDAC inhibitors trichostatin A (TSA) and NaB restore responsiveness of the *CYP1A1* gene towards TCDD and 3-MC in fibroblasts (Gradin et al., 1999; Nakajima et al., 2003), we treated our fibroblasts with NaB alone or in combination with B(a)P. As shown in figure 6A, NaB exposure displaced the AhRR from the XRE of the *CYP1A1* gene. This effect was more pronounced in cells which were co-treated with NaB and B(a)P. Comparable results were obtained from ChIP assays performed in HeLa cells (Fig. 6B). Treatment of this cell line with NaB confirmed the sensitivity of the observed constitutive AhRR/XRE association toward the HDAC inhibitor.

In order to assess the responsiveness of *CYP1A1* in primary fibroblast and HeLa cells, we performed RT-PCR analyses after treating the cells with NaB and a combination of NaB and B(a)P. In comparison to the control values, NaB enhanced CYP1A1 mRNA 2-fold in fibroblasts and 30-fold in HeLa cells (Fig. 6C). Co-treatment with B(a)P resulted in a ~200-fold induction of CYP1A1 mRNA in fibroblasts and an about 400-fold enhancement of CYP1A1 mRNA in HeLa cells (Fig. 6C). It has to be mentioned, that neither NaB nor the combination of NaB/B(a)P led to changes in AhRR mRNA expression in fibroblast and HeLa cells (data not shown). Thus, the data from the ChIP analyses and the PCR-experiments support the previous notion that the non-responsiveness of *CYP1A1* in primary fibroblast and HeLa cells is mediated by the inhibitory activity of the AhRR and that HDACs are involved in this gene regulation.

Discussion

The human *AhRR* gene is located on chromosome 5p15.33 and distributed in a total sequence length of approximately 130kb. The gene consists of twelve exons with the ATG start codon in exon 2. The DNA binding bHLH domain is encoded by exon 3 and the PAS domain by exons 5 and 6 (Cauchi et al., 2003). We identified four putative XRE sites in intron I of the *AhRR* gene and subsequently cloned this 1.025 kb DNA fragment into a luciferase reporter plasmid (Fig. 1A). By means of reporter gene assays, generation of different deletion constructs and mutational analysis, we were able to show that the XRE-3 has functional activity as indicated by 3-MC responsiveness (Fig. 1B, 1C). This XRE-3 is located at position +17.507 and contains the classical XRE core sequence GCGTG.

In order to confirm the functional importance of the identified XRE-3, we analyzed binding of AhR, ARNT and AhRR to this XRE. ChIP analyses obtained from HepG2 cells revealed a constitutive association of these three proteins with XRE-3 confirming an involvement of the AhR in regulation of constitutive *AhRR* gene expression (Fig. 2). Noteworthy, we observed a strong binding of AhR at XRE-3 at time-point zero. However, in several ChIP experiments using untreated cells, we noticed variations in AhR/XRE binding intensity (data not shown), suggesting a periodic association of AhR with XRE-3 during regulation of constitutive *AhRR* transcription. A similar constitutive AhR/XRE-binding was reported for the *c-myc* gene (Yang et al., 2005). Upon treatment of the cells with 3-MC for the indicated time-points, we observed an oscillating binding-pattern of AhR, ARNT and AhRR to the respective XRE-3 site of the AhRR intron I (Fig. 2). A similar promoter on-and-off cycling was observed for AhR and nuclear coactivator proteins at the *CYP1A1* enhancer (Hestermann and Brown, 2003). This oscillation pattern was accompanied by binding of RNA polymerase II, indicating a high flexibility of the transcription machinery. Although, the time-course displays only minor changes in AhRR signal density, the observation that the AhRR binds to its own XRE is exciting, since it suggests a possible auto-regulatory loop for *AhRR* transcription.

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In contrast to the human *AhRR*, the murine *AhRR* gene is regulated by three XRE sites located in the 5'-upstream region of the gene. In addition, three putative GC-boxes have been identified within the enhancer region of the murine *AhRR* (Baba et al., 2001). Mutational analyses of these GC-box sequences resulted in a lower constitutive and inducible expression of the *AhRR* (Baba et al., 2001). In this study, we identified a putative GC-box 8 bases 5'-upstream of the functional XRE-3 (Fig. 1A). Our data indicate a strong link between the presence of the GC-box and XRE-3, since the deletion of both, the GC-box and XRE-3, resulted in a reduction of basal and inducible luciferase activities (Fig. 1B, *pAhRR-Δ4*). To clarify if this GC-box adjacent to XRE-3 is of functional relevance, we blocked this sequence with the selective GC-binding inhibitor MitA and subsequently investigated AhRR reporter gene activity and mRNA expression. The data from the *pAhRR-Δ5* reporter gene assays revealed that MitA-mediated inhibition of GC-box activity interferes with both, the basal and inducible XRE-driven luciferase activity (Fig. 3). This finding points to a cooperative function of the AhR and GC-box recognizing transcription factors in regulation of AhRR expression. RT-PCR analyses performed in HeLa cells treated with MitA confirmed the luciferase experiments. In the presence of MitA, the expression of AhRR mRNA decreased in a time-dependent manner (Fig. 4A). This decrease was accompanied by an increase of CYP1A1 mRNA, which was more pronounced after co-treatment with 3-MC and MitA (Fig. 4B).

A cooperative activity between the GC-box binding protein Sp1 and the AhR/ARNT complex was reported for the regulation of human *CYP1A1* and *CYP1B1* (Kobayashi et al., 1996; Tsuchiya et al., 2003b). It was shown that the bHLH/PAS domains of the AhR/ARNT complex interact with the Sp1 zinc-finger motif (Kobayashi et al., 1996). In contrast, a repressive role in the regulation of *CYP1A1* was reported for the GC-box binding, Sp1-related transcription factors BTEB (basic transcription element binding) 3, BTEB 4 and gut-enriched krüppel-like factor (Imataka et al., 1992; Zhang et al., 1998; Kaczynski et al., 2002). The

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observed up-regulation of *CYP1A1* by MitA may be either due to interference with the GC-box in the *AhRR* enhancer region and a consequentially decreasing AhRR synthesis or to a displacement of repressive-acting Sp-like molecules from the *CYP1A1* enhancer.

In order to test whether Sp1 has a regulatory function in the constitutive expression of the *AhRR*, we performed transient transfection experiments with siRNA targeted against Sp1 in HeLa cells. No significant effect on the expression of *AhRR* was found (data not shown) indicating that Sp1 is not the dominant molecule in regulation of the constitutive *AhRR* gene expression. Therefore, other GC-box binding proteins may account for the observed effects.

As described in the literature, HepG2, HeLa and primary fibroblasts differ markedly in their basal expression levels of *AhRR* and their responsiveness toward AhR agonists (Gradin et al., 1993; Gradin et al., 1999; Iwanari et al., 2002; Nakajima et al., 2003; Tsuchiya et al., 2003a). Therefore we wondered whether the expression level of the AhRR controls the non-responsiveness of *CYP1A1* towards AhR agonists. ChIP analyses performed in high AhRR-expressing primary fibroblasts revealed a strong constitutive binding of the AhRR to XRE motifs of the *CYP1A1* gene, which was not affected by B(a)P treatment (Fig. 6A). However, exposure of the cells to the HDAC inhibitor NaB caused a significant decrease in AhRR/XRE binding, which was more pronounced after co-treatment with B(a)P. In agreement with this finding, the constitutive XRE binding of the AhRR observed in HeLa cells was completely diminished after NaB exposure (Fig. 6B). Similar results were observed with TSA (Lorenz Poellinger, personal communication). The results from the ChIP assays suggest that a high amount of constitutive AhRR controls the inducibility of AhR target genes. These data were confirmed by RT-PCR analyses of fibroblast and HeLa cells, treated with NaB alone or in combination with B(a)P. As shown in figure 6C, both types of treatment led to an increase of the constitutive and inducible expression of *CYP1A1*. Similar findings were described for

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CYP1A1 inducibility in TSA-treated fibroblasts (Gradin et al., 1999) and HeLa cells (Nakajima et al., 2003).

The exact mechanism by which NaB interferes with HDAC activities is not known (Davie, 2003). It was reported that NaB disturbs the conformation of a nuclear HDAC/co-repressor complex (Downes et al., 2000). The structural integrity of such complexes is important for modulation of the chromatin structure and consequently for gene expression (Grozinger and Schreiber, 2002). In uninduced murine hepatocytes, NaB treatment led to histone hyperacetylation at the *CYP1A1* locus. In these cells, the basal *CYP1A1* expression is repressed due to an interplay of HDAC and DNA-methyltransferase molecules, representing a strictly epigenetic mechanism of *CYP1A1* silencing (Schnekenburger et al., 2007). Therefore, the 3-MC responsiveness of fibroblast and HeLa cells after NaB exposure can be the consequence of an enhanced histone acetylation at the *CYP1A1* gene. However, it was reported that the non-responsiveness of *CYP1A1* in fibroblasts depends on a constitutively expressed ARNT-bound factor. The authors speculate that this unknown repressor is able to recruit HDAC molecules to the XREs of *CYP1A1* (Gradin et al., 1999). From our data we hypothesize that the AhRR is part of such a regulatory co-repressor complex, which recruits HDAC enzymes and subsequently represses the transcription of genes like *CYP1A1*. Although, the precise function of AhRR in *CYP1A1* gene regulation is not fully understood, our siRNA results (Fig. 5) let us conclude, that the AhRR takes part in the regulation of constitutive and inducible *CYP1A1* expression.

The AhRR-mediated control of *CYP1A1* transcription in primary fibroblasts may be of biological relevance. Fibroblasts are important for wound healing, synthesis of extracellular matrix components and thus essential for the integrity of many organs and tissues (Heckmann and Krieg, 1989). High AhRR levels may be part of a cellular defense mechanism which keeps the expression of *CYP1A1* down and thereby protects these cells from toxic metabolites and reactive oxygen species (Haarmann-Stemmann and Abel, 2006).

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In summary, we cloned a regulatory fragment of the human *AhRR* gene containing one functional XRE motif and one functional GC-box. The observed association of the AhRR protein with its own XRE site indicates an auto-regulatory loop of *AhRR* expression. In addition, we have shown that the non-responsiveness of fibroblasts and HeLa cells with regard to *CYP1A1* induction by AhR agonists is attributed to binding of the AhRR to the respective XRE sites of the gene. Treatment with the HDAC inhibitor NaB led to dissociation of the AhRR complex from the XRE sites, resulting in recovery of *CYP1A1* inducibility. The function of the AhRR in regulation of *CYP1A1* transcription is confirmed by RNA interference experiments, showing that a transient AhRR knock-down is accompanied by an increased *CYP1A1* mRNA expression. We propose that the AhRR plays an important role in triggering the expression and induction of AhR-regulated genes like *CYP1A1*. The precise molecular mechanisms, how the AhRR regulates XRE-driven gene expression remains to be further elucidated.

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Legends for figures

Figure 1. Identification of functional XRE sites within intron 1 of the human AhRR gene. A) Schematic structure (not scaled) of the human AhRR gene and localisation of the cloned regulatory region of intron 1. B) Reporter gene assays. HepG2 cells were transfected with 2 μ g of *pGL3-basic*, *pAhRR*, each of the generated deletion constructs *pAhRR- Δ 1* to - Δ 5 and the *pAhRR-mut* construct. 24h later cells were treated for 48h with 10 μ M 3-MC or solvent DMSO (final concentration 0.1%). Firefly luciferase activities were measured and corrected to Renilla luciferase activities as described in experimental procedures. Relative luciferase activities are presented as fold of the solvent treated empty vector *pGL3-basic*. C) Effect of MNF on the 3-MC inducible luciferase activity of *pAhRR- Δ 5*. Transfected HepG2 cells were treated for 48h with DMSO (final concentration 0.1%), 10 μ M 3-MC and a combination of 10 μ M 3-MC / 10 μ M MNF. Relative luciferase activities are shown as fold of solvent control. * = significantly different from solvent control, † = significantly reduced in comparison to the 3-MC treated sample. Data are shown from three independent experiments, samples were analyzed in triplicates.

Figure 2. Chromatin immunoprecipitation of a XRE-3 containing AhRR fragment. HepG2 cells were treated for 0, 10, 20, 30, 40, 50, and 60min with 10 μ M 3-MC. Chromatin samples were immunoprecipitated with antibodies against AhR (*row 1*), ARNT (*row 2*) and AhRR (*row 3*). The input signals are shown in *row 4*, the IgG control in *row 5*.

Figure 3. Inhibition of basal and inducible luciferase activity by MitA in HepG2 cells transiently transfected with the *pAhRR- Δ 5* plasmid. Cells were transfected with 2 μ g of the *pAhRR- Δ 5* deletion construct and incubated overnight. Cells were treated for 24h with 10 μ M 3-MC, 150nM MitA or in combination 10 μ M 3-MC / 150nM MitA. Luciferase activities were

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measured as described in material and methods. The results are shown as fold of untreated control. * = significantly different from the solvent control, † = significantly reduced in comparison to the 3-MC treated sample. Number of experiments and replicates as described in the legend of figure 1.

Figure 4. Effect of MitA exposure on constitutive and inducible expression of AhRR and CYP1A1 mRNA in HeLa cells. AhRR and CYP1A1 mRNA levels were quantified using real-time PCR and normalized to β -actin transcripts. Expression levels are given as fold of untreated control. **A)** Influence of MitA on constitutive mRNA expression of AhRR and CYP1A1. Cells were treated for 1.5, 3, 4.5, 6, and 12h with 150nM MitA. **B)** Effect of MitA on inducible AhRR and CYP1A1 mRNA expression. Cells were treated for 12h with 10 μ M 3-MC alone or in combination with 150nM MitA. * = significantly different from solvent control (DMSO). Number of experiments and replicates as described in the legend of figure 1.

Figure 5. Effect of a transient AhRR knock-down on CYP1A1 gene expression in HeLa cells. HeLa cells were transfected with 100nM siRNA using the Fugene reagent. 24 and 48h after transfection mRNA levels of AhRR and CYP1A1 were determined by RT-PCR analyses and normalized to β -actin transcripts. For the 3-MC-studies, cells were transfected for 24h with siRNA and then treated with DMSO or 10 μ M 3-MC for 24h. Treatment 24h. Expression levels are given as fold of non-silencing controls or DMSO-treated non-silencing controls. * = significantly different from non-silencing control, † = significantly reduced in comparison to the DMSO-treated sample. Number of experiments and replicates as described in the legend of figure 1.

Figure 6. Influence of the HDAC inhibitor NaB on AhRR/XRE binding and CYP1A1 expression in fibroblast and HeLa cells. A) Chromatin immunoprecipitation of the CYP1A1

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enhancer region with an AhRR antibody in primary fibroblasts. Cells were treated for 16h with 10 μ M B(a)P, 10mM NaB or a combination of 10 μ M B(a)P and 10mM NaB. *Lane 1*: controls, *lane 2*: B(a)P, *lane 3*: NaB and *lane 4*: B(a)P / NaB. B) Effect of NaB on AhRR/XRE binding in HeLa cells. Cells were treated with 10mM NaB for 16h and CHIP analysis was performed as indicated. *Lane 1*: control, *lane 2*: NaB. C) Influence of NaB on constitutive and inducible CYP1A1 mRNA expression. Cells were treated with 10mM NaB and 10 μ M B(a)P / 10mM NaB for 24h. CYP1A1 mRNA expression was measured by RT-PCR and normalized to β -actin transcripts. Expression levels are given as fold of solvent controls. * = significantly different from control. Number of experiments and replicates as described in the legend of figure 1.

Figure 1A

A.

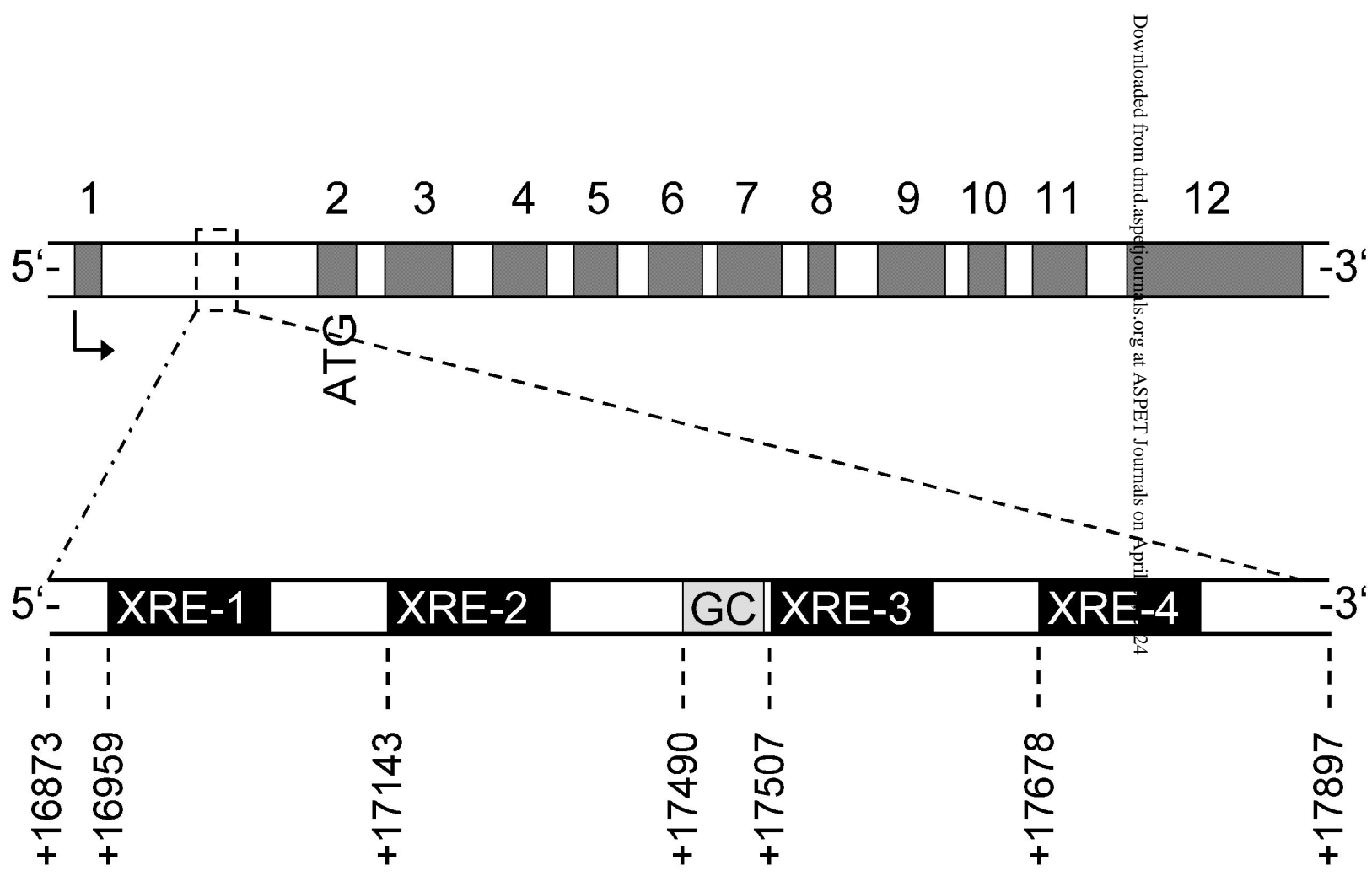


Figure 1B

B.

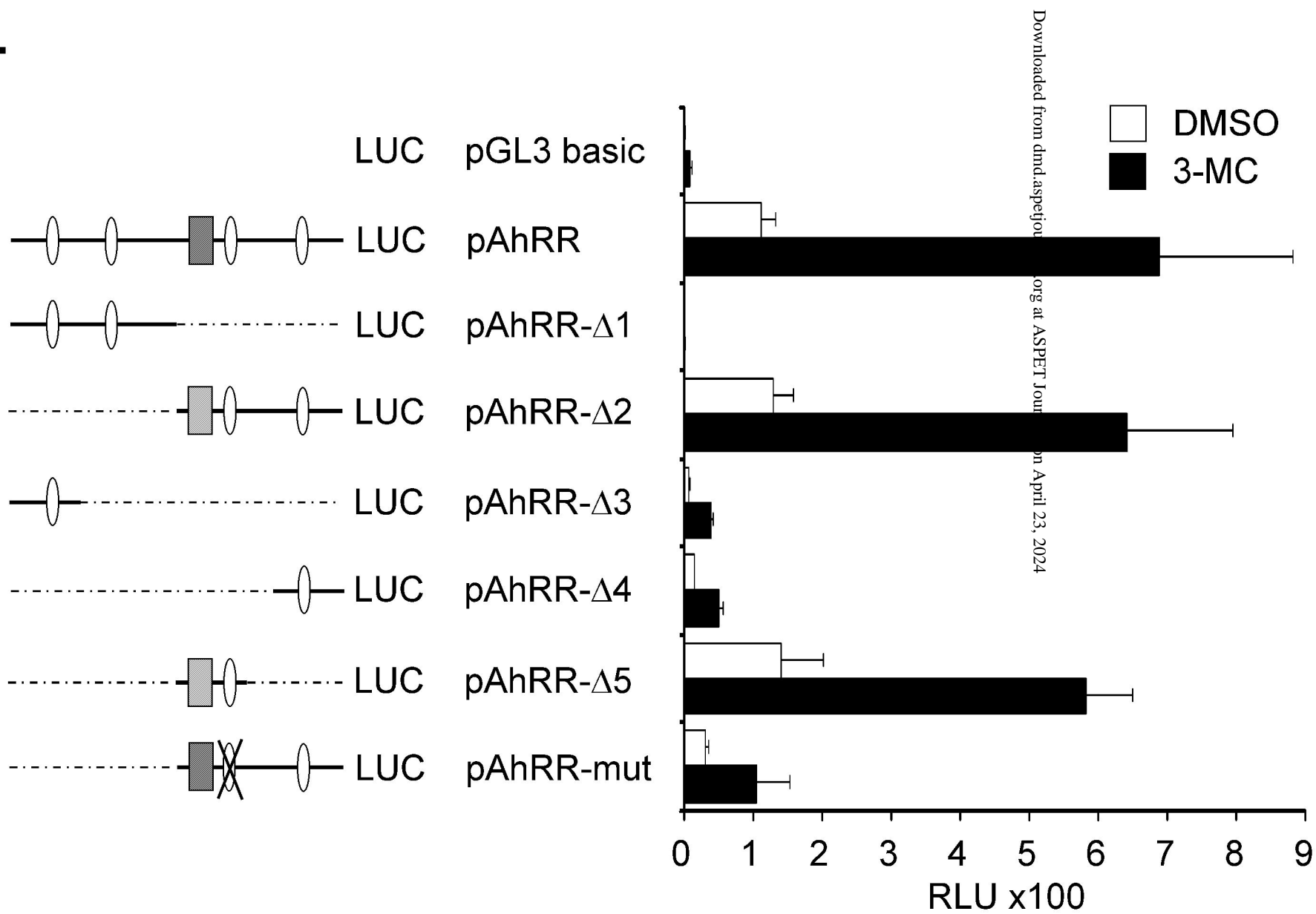


Figure 1C

C.

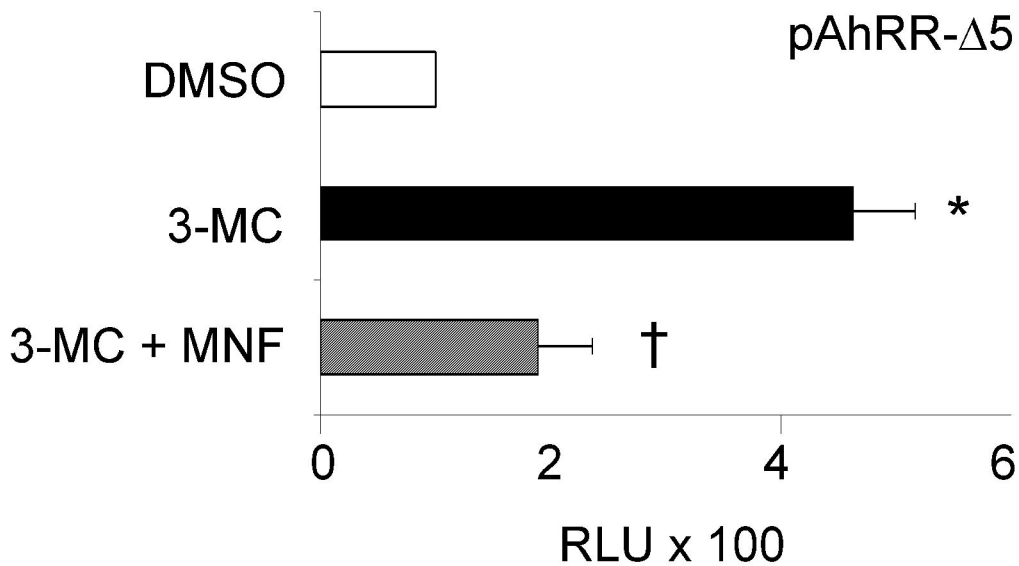


Figure 2

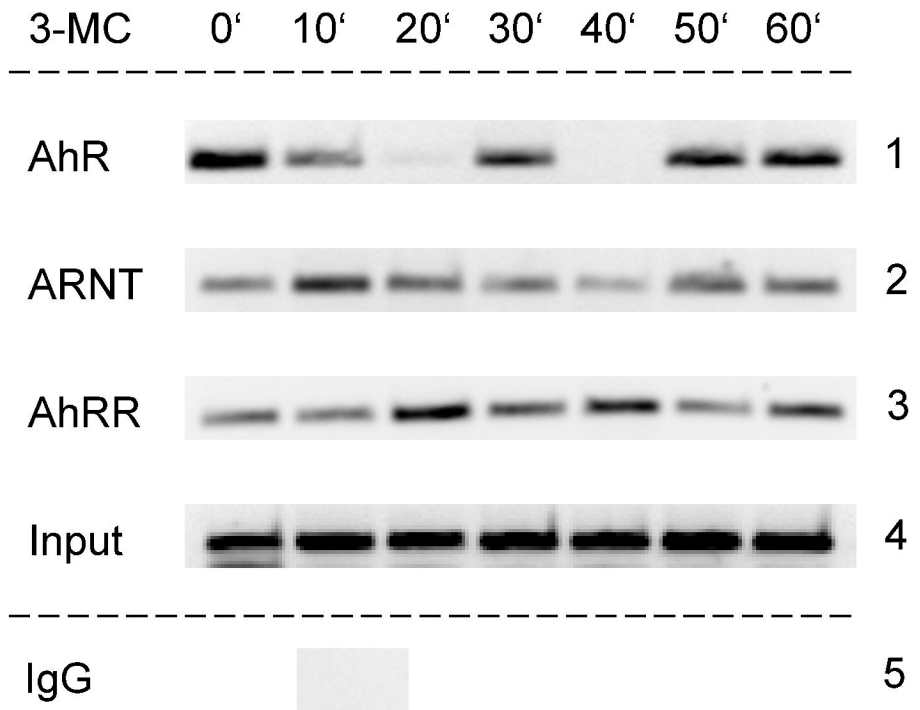


Figure 3

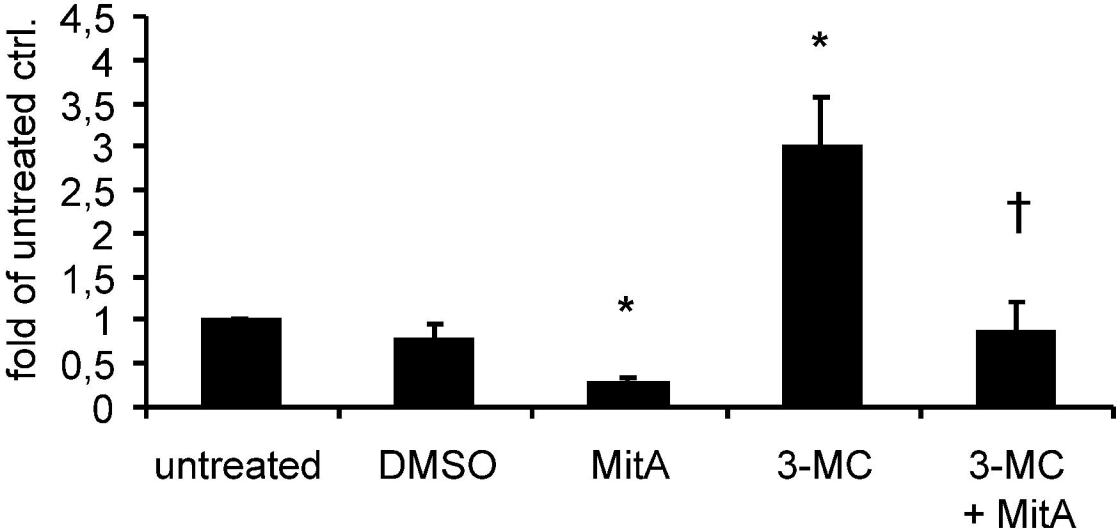
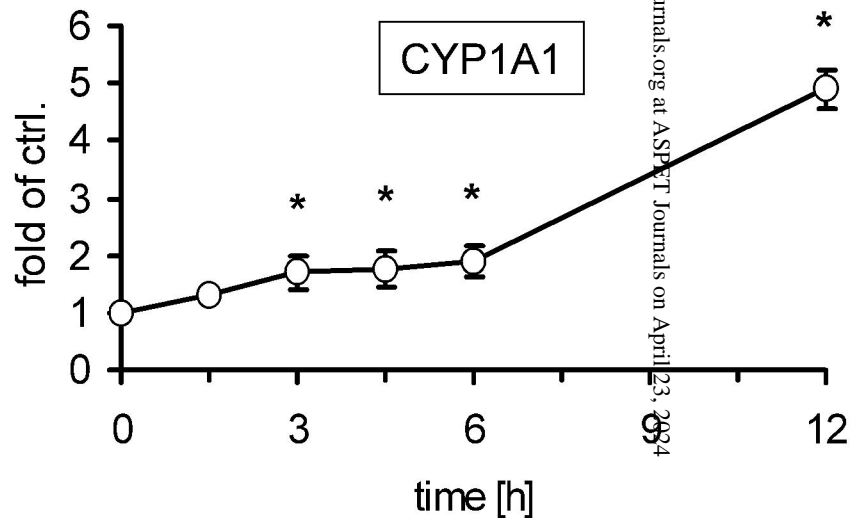
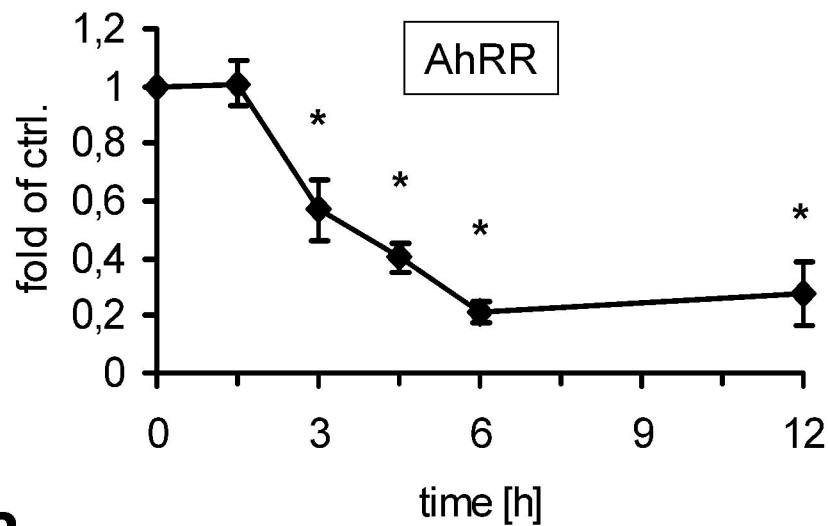


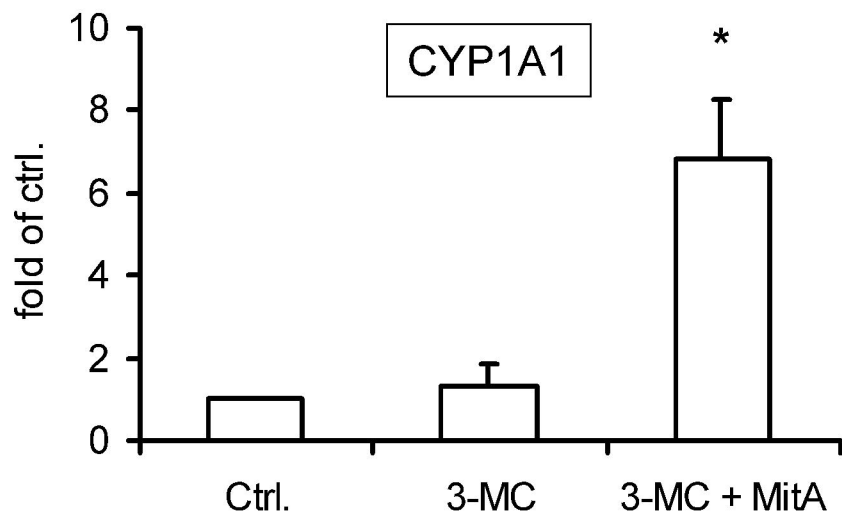
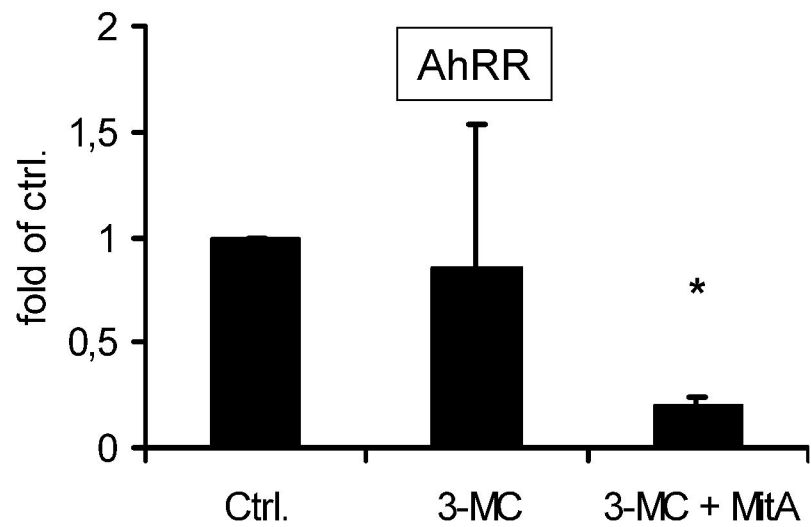
Figure 4

A.



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B.



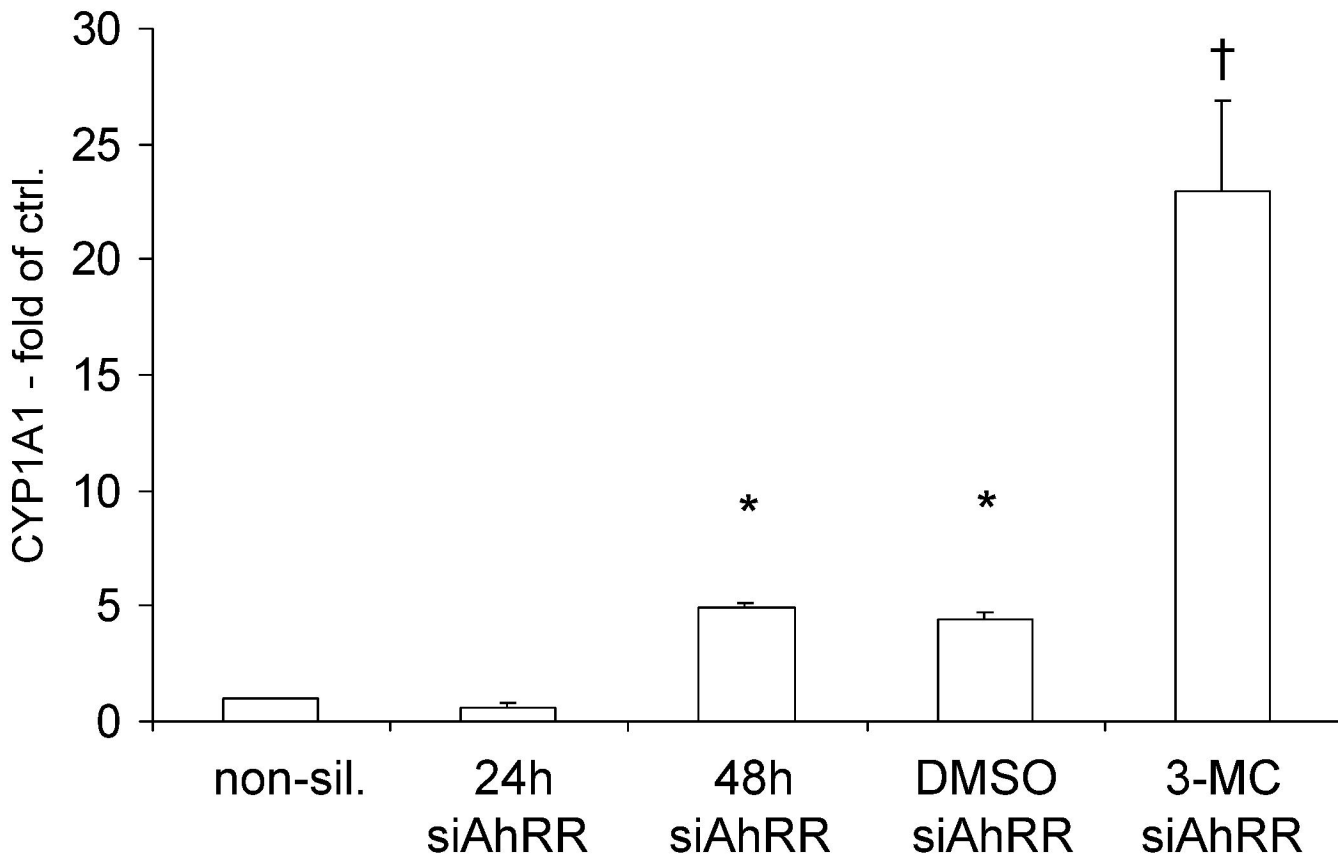
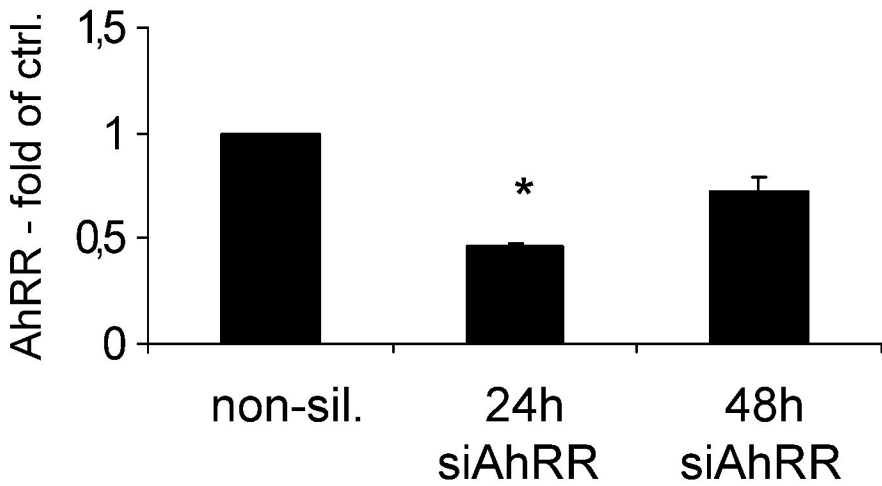


Figure 6

A.



AhRR

Input

IgG

B.



AhRR

Input

IgG

C.

